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BIOLOGICAL PROPERTIES OF THE A_{22} VARIANT

OF HOOF AND MOUTH DISEASE VIRUS

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BIOLOGICAL PROPERTIES OF THE A_{22} VARIANT OF HOOF AND MOUTH DISEASE VIRUS

by

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Research of recent years has confirmed the relatively high mutability and adaptive capability of hoof and mouth disease virus. Considerable successes have been achieved after suckling mice and newborn rabbits, which are very susceptible to hoof and mouth disease, began to be used as experimental animals, as well as tissue cultures.

The method of changing the hereditary properties of viruses by means of passaging in tissue cultures at reduced temperatures merits the special intention of investigators. During these passages the capacity of the viruses to propogate is altered, which is accompanied by changes in other biological properties, including pathogenicity. On the basis of theoretical concepts, introduced by A. L'vov (1960, 1962), by means of cultivation of hoof and mouth disease virus at reduced temperatures, attenuated strains which are useful practically have been obtained.

Research of Wittmann, also Asso and others (1964-1966) the different degrees of propagation of attenuated and virulent hoof and mouth disease virus strains at high and low temperatures, and also the virulence for susceptible animals were revealed.

We have studied certain immunobiological and physico-chemical properties of original (virulent) and modified strains of hoof and mouth disease A_{22} virus, obtained in the process of passaging in the organism of non susceptible animals and in a culture of cells of calf kidney at reduced temperature.

Materials and Methods

Hoof and mouth disease virus A_{22} (A.SSSR, 550/65): epizootic aphthous virus from horned cattle, lapinized virus of the 6th and 149th passages.

Animals: horned cattle weighing 240-250 kg, guinea pigs - 500-700 g, 2-day old rabbits, 45-day old mice, white mice weighing 20-25 g.

(Tissue culture): primary cultures of cells were obtained from calf kidneys (CK) 6-8 months old according to the generally accepted method. Cultures were grown in test tubes and in flasks of various volumes at a temperature of 37° for 6-8 days in a medium with 0.5% hydrolysate of lactalbumin in Henks' solution (LHH) with 10% normal cattle serum. Before infection the cultures were washed with Henks' solution. In order to cultivate virus LHH medium at pH 7.6 without serum was used.

(Titration of virus): The infectious titer of the virus was determined by cytopathogenic effect (CPE) in test cultures and in mice. Virus was titrated at 10-fold dilutions with an interval of 1 logarithm. Cultures in test tubes were infected with a suspension of virus at a dosage of 0.1 ml and were brought up to 0.9 ml of supporting LHH medium. Infected cultures were incubated at temperatures of 41, 37, 28 and 24°. The experiments were finally registered on the 5th, 7th day. Mice were infected with a suspension of virus in a volume of 0.1 ml. Results were studied for 7 days. The virus titer was calculated by the method of Reed and Mench.

Neutrallzation and Seroprotection

The titer of virus neutralizing antibodies in blood sera of animals was determined according to the neutralization reaction in tissue culture and the index of seroprotection in mice. Before setting up the reaction, sera were heated at 56-58°. In the neutralization reaction two-fold dilutions of sera in Henks' solution were mixed with various volumes of virus containing 100 $TTsD_{50}$/ml. The mixture was retained for contact at 37° for one hour.

Cultures and test tubes were infected at a dose of 0.2 ml and brought up to 0.8 ml with sustaining LHH 80 m at pH 7.6. The results were studied for a period of 3 days. In order to determine the index of protection mice were injected subcutaneously with 0.1 ml of undiluted test serum, and on the following day virus was titrated parallely in mice protected and not protected by the serum. The index of protection was calculated according to the difference in virus titers in mice which were and were not treated with serum.

The antigenic properties of the virus were studied in the compliment fixation test with homologous and standard type-specific sera.

For genetic characteristics of the virus in the process of passaging, the following markers were used: pathogenicity for guinea pigs (Vg), mice (Vm), rabbits (Vl) and large cattle (Vb), thermoresistance (T_{50}), stability at pH 6.5 (pH), capacity for propagation in a culture of CK cells at 41, 37, 28, and 24° (rct_{41}, rct_{37}, rct_{28}, rct_{24}), the dimensions and time of formation of negative colonies under agar (S).

Pathogenicity of the virus was determined by infection of guinea pigs, newborn rabbits, mice and large cattle. Guinea pigs were infected intracutaneously at the plantar surface of the hind paws, rabbits - subcutaneously with 1 ml, mice - subcutaneously with 0.1 ml, cattle - subcutaneously with 1-2 ml.

The T_{50}-criterion - the test virus - containing suspension was heated in a water bath at 50° for 30 minutes (temperature fluctuation $\pm$ 0.2°), abruptly cooled and the infectious activity of the virus was determined by the method of titration in mice or in tissue culture;

pH-criterion - a suspension of the virus was extracted 30 minutes at 4° with phosphate buffer pH 6.5, then the pH of the medium was adjusted to 7.6 with sodium bicarbonate solution and titrated in mice or in tissue culture. A dilution of the virus in phosphate buffer at pH 7.6 and its titration in mice or in tissue culture served as the control.

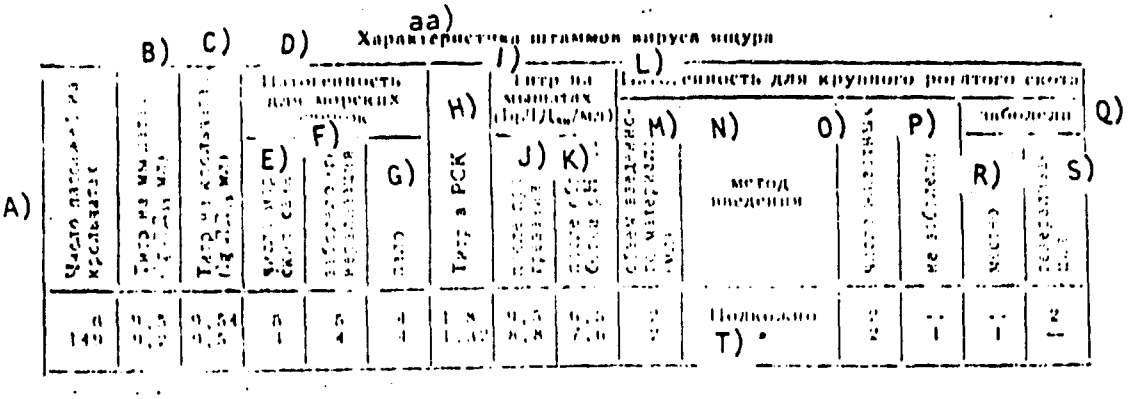
rct-criterion - determined according to CPE by the method of titration of virus in a culture of cells at various temperatures.

Results of Research

The pathogenic virus for further study and comparison of its properties with a modified strain selected was hoof and mouth disease virus A_{22}, passaged 22 times in rabbits, then renewed in cattle, again passaged 10 times in rabbits, renewed in cattle and passaged 6 times in rabbits. This virus is a commercial strain for preparation of hydroxy aluminum formol vaccine.

This commercial virus strain, passaged 117 times in addition in rabbits and designated as the 149th passage (Table 1) was selected as the original virus for subsequent passaging in tissue culture and for obtaining a modified strain.

Table 1 shows that virus of the 6th and 149th passages was virulent for mice and rabbits, propagated in the organisms of these animals at high titer (9.5 log LD_{50}/ml); caused disease in guinea pigs in the generalized form with 100% fatality; was insensitive to the effect of temperature at 50° for 30 minutes (T_{50-}); weakly sensitive to the pH of the medium 6.5. Antigens were specific and active in the compliment fixation tests with homologous serum at dilutions of 1:8 and 1:32.



Key: aa) Characteristics of Hoof and Mouth Disease Virus Strains; A) number of passage from rabbits; B) titer in mice (log LD/ml); C) titer in rabbits (LD_{50}/ml; D) pathogenicity for guinea pigs; E) number of guinea pigs; F) affected generalized); G) died; H) titer in compliment fixation test; I) titer in mice (log LD_{50}/ml; J) after heating; K) after treatment at pH 6.5; L) pathogenicity for cattle; M) volume of injected material (ml); N) method of injection; O) number of animals; P) not affected; Q) affected; R) locally; S) generalization; T) subcutaneously.

After subcutaneous injection of the centrifuged virus suspension at a dilution of 1:10 in a volume of 2 ml with 2 head of cattle, hoof and mouth disease was observed in them in a generalized form. From data of a control at biofactories it is also known that this virus causes disease of cattle with generalization of the process upon injection in the tongue. Testing of the virus of the 147th passage in cattle showed that it had somewhat reduced its pathogenic properties (of 2 animals 1 was affected by formation of aphthae on the mucous lining of the tongue.

For further modification the virus of the 149th passage in rabbits was adapted to a culture of CK cells. At the first passage it already caused complete degeneration of cells after 48 hours at a culturing temperature of 37°. From the 2nd to the 20th passage (virus was cultivated at 37°) the cytopathogenic effect of the virus was observed after 18-20 hours. At the 21st-25th passage the virus was cultured at 28°, and the calve's CPE began after 48 hours.

From the 25th passage cultivation of the virus at 24° was begun but after 72 hours only 10-15% of the cells were affected, and virus of the 26th passage did not cause CRE. However, after carrying out an additional 3 passages at 26°, reproduction of the virus in tissue culture at 24° was enhanced and a further possibility of passaging virus at this temperature appeared (Table 2).

aa) Характеристика некоторых свойств вируса ящура в процессе его пассирования в культуре клеток ПГ

A)	B)	C)	D)	E) Патогенность для морских свинок					L)		O)	
				F)	G)	H) заболели		K)	M)	N)		P)
						I)	J)					
10	37	5,4	96 — 102	3	—	1	2	—	5,2	0	1:2	
35	37	5,4	96 — 102	3	—	—	3	1	5,4	0	1:2	
45	24	4,3	0	3	1	—	3	1	2,7	0	н/р	
60	24	1	0	3	7	1	2	—	1	0	н/р	

Q) Обозначения: 0 — животные не заболели, н/р — неразведенная вирусосодержащая культуральная жидкость.

Key: aa) Characteristics of Certain Properties of Hoof and Mouth Disease Virus in the Process of its Passaging in a Culture of CK cells: A) number of passages B) incubation temperature (°); C) titer in mice (log LD_{50}/ml); D) time of death of rabbits (hours); E) pathogenicity for pigs; F) number of animals; G) not affected; H) affected; I) locally; J) generalization; K) died; L) titer in mice (log LD_{50}/ml); M) after heating; N) after treatment at pH 6.5; O) titer in compliment fixation test; P) undil. Q) Key: 0-animals not affected; undil-undiluted virus containing culture fluid.

In the experiments no substantial differences were noted in the properties of virus passaged for 10-20 passages in a culture of cells at 37°. However, virus titer in mice in comparison with the original strain was reduced by 9.5 to 5.25-5.4 log LD_{50}/ml; death of rabbits began 96-102 hours after their injection with undiluted virus containing culture fluid; in guinea pigs local and generalized forms of hoof and mouth disease were detected. The virus reduced the sensitivity to the acid reaction of the medium, but remained insensitive to heating at 50° for 30 minutes (T_{50-}).

Study of the biological properties of virus of the 35th, 45th and 60th passages, cultured at 24°, showed that its pathogenicity for mice, rabbits, and guinea pigs decreased regularly in the process of passaging. At the level of the 60th passage the virus titer for mice was less than log LD_{50}/ml; the virus did not cause disease in rabbits and in only one of 8 guinea pigs formed an abortive apthae on one paw; the virus possessed T_{50-} and pH criteria. Cultural antigens of the 10th, 20th, 35th, 40th, and 60th passages were specific and active in the compliment fixation test with homologous serum (undiluted and at a titer of 1:2).

Results of propagation of the virus of the 10th, 20th, 35th, 45th, and 60th passages at 41, 37, 28, and 24°, was also its capacity to form negative colonies (MC) are presented in Table 3.

aa) Данные о размножении вируса ящура при различных температурах и способности образовывать негативные колонии

A) Пассаж вируса	B) Титр вируса в культуре клеток при различной температуре (ТЦД$_{50}$/мм)				C) Характер НК	D) Время появления НК (часы)	E) Температура (°)	F) Размеры (мм)
10								
20								
35								
45								
60								

Key: aa) Data on Propogation of Hoof and Mouth Disease Virus at Various Temperatures and Capacity to Form Negative Colonies; A) passage of virus; B) virus titer in tissue culture at various temperatures ($TTsD_{50}$/mm); C) character of MC; D) titer appearance (hours); E) incubation temperatures (°); F) dimensions mm).

In the process of passaging at reduced temperatures the capacity of the virus to propagate at 41 and 37° gradually decreased (after the 45th passage the virus did not propagate at 41°, and by the 60th passage at 41 and 37°). The capacity of the virus to propagate at 28 and 24° increased and the titer of the 60th passage was equal to 7 log TsT_{50}/ml).

Thus, at the level of the 10th passage the virus possessed rct_{41+}, rct_{37+}, rct_{28+}, rct_{24+}; the 20th - rct_{41+}, rct_{37+}, rct_{28+}, rct_{24+}; the 35th - rct_{41+}, rct_{37+}, rct_{28+}, rct_{24+}; the 45th - rct_{41-}, rct_{37+}, rct_{28+}, rct_{24+}; the 60th passage - rct_{41-}, rct_{37-}, rct_{28+}, rct_{24+} criteria.

Virus of the 10th and 20th passages at 37° caused formation of large MC (with dimensions 4-5 mm) after 96 hours. Virus of the 35th and 45th passages at 37° formed MC (with dimensions 2-3 mm) after 72 hours. Virus of the 60th passage had lost the ability to form MC at 37° and had acquired the ability to cause formation of MC with dimensions 1-1.5 mm) at 28° within 48 hours.

In Table 4 data are presented on testing of immunogenic properties of hoof and mouth disease virus passaged in a culture of CK cells in cattle.

The viruses injected subcutaneously into the animal in the region of the middle third of the neck at doses of $2 \cdot 10^{6.8}$ - $2 \cdot 10^{7.2}$ $TTsD_{50}$/ml. They were observed for a period of 15 days. In one out of three calves vaccinated with virus of the 45th passage, on the 5th day after vaccination the temperature rose to 40.3° and on the 7th day abortive apthae developed on the mucous membrane of the tongue; in the 2nd one - on the 15th day erosion was observed on the mucous lining of the tongue and gums without an increase in temperature. In both animals a depressed state, with salivation and disruption of food intake were not observed. One animal remained healthy.

Virus of the 60th passage was injected into three head of cattle; all animals remained healthy.

The titer of the virus neutralizing antibodies and sera according to the neutralization reaction in tissue culture and according to the seroprotection reaction in mice on the 15th day after vaccination was 1:84 - 1:1024 or < 7 log.

Control infection was carried out 15 days after vaccination with apthous virus from cattle with a titer of 9.1 log LD_{50}/ml at a dilution of 1:500 by the method of smearing on the mucous membrane of the tongue. All vaccinated animals were healthy for a period of ten days (the period of observation). Control animals were stricken with hoof and mouth disease with generalization of the process.

Discussion

Prolonged passaging of hoof and mouth disease virus in the organism of naturally immune animals leads to a change in its biological properties. However, despite multiple tests, it was not possible to obtain strains by this method which would posses along with apathogenicity sufficiently immunogenic properties. Moreover, as a result of such passaging the virus acquires the properties of myotropicity and cardiotropicity [A. Paraff, et al, 1961; P. Prunet, 1964].

From the work of Asso et al [1966] it is known that the myotropic and cardiotropic effect of such strains may be eliminated by means of their passaging in cultures of primary tripsonzied kidney tissues of cattle or hogs.

Extremely encouraging results have also been obtained by investigators [G. Wittmann, 1964; L. Goldsmit, E. Barzili, 1964; Asso, et al, 1966], using the principles of selection and modification of hoof and mouth virus strains by means of culturing them at a temperature below that at which the virus causes disease in naturally susceptible animals in their experiments.

We have presented a perspective for the purpose of obtaining a harmless and immunogenic strain of the virus to employ prolonged passaging of the virus in the organism of naturally immune animals and in a culture of cells at 37 and 24°.

Table 4

aa) Данные об испытании на крупном рогатом скоте иммуногенных свойств вируса ящура

A) Пассаж вируса	B) Число животных	C) Объем введенного материала (мл)	D) Метод введения	E) Контроль на вирулентность: F) не заболели	G) заболели: H) местно	G) заболели: I) генерализация	J) Контроль на активность: F) не заболели	J) Контроль на активность: G) заболели: H) местно	J) Контроль на активность: G) заболели: I) генерализация	K) Титр антител после вакцинации: L) реакция нейтрализации	K) Титр антител после вакцинации: M) реакция серопротекции (lg)
45	3	2	N) Подкожно	1	2	—	2	—	—	1:81 1:162	2 1,9
60	3	2	O) то же	3	—	—	3	—	—	1:208 1:160 1:10,6	≤2 3,4

Key: aa) Data on Testing of Immunogenic Properties of Hoof and Mouth Disease Virus in Cattle; A) passage of virus; B) number of animals; C) volume of injected material (ml); D) method of injection; E) control on avirulence; F) not affected; G) affected H) locally; I) generalization; J) control on activity; K) antibody titer after vaccination; L) neutralization reaction; M) seroprotection reaction (log); N) subcutaneously; O) ditto.

In our experiments as a result of prolonged passaging in the organisms of newborn rabbits the virus also remained highly pathogenic for naturally immune animals, decreased its pathogenicity in relation to cattle and acquired the property of myotropicity.

Further passaging of the virus in a culture of CPE cells at 37°, and then at 24° led to reduction in pathogenicity of the virus for rabbits, mice, and guinea pigs with retention of immunizing and antigenic properties. The ability of the virus to propagate at 41, 37, 24, and 28° was altered.

The virus remained at high titer to propagate at 24 and 28° and lost the ability to produce CPE at 41 and 37° which is in agreement with research of Asso et al and Wittmann [1964-1966], which showed that in proportion to the depression in the adaptation of the virus, it loses its primary ability to propagate at 40 and 37° and acquires the ability to propagate at low temperatures.

According to data of Korkh (1), Asso and Aunaud (1) [1966], the sensitivity of the virus to heating at 50-55° is an indication of the virulence or avirulence of the strain. It was established that attenuated strains are characterized by high sensitivity to the temperatures indicated. In our experiments, in proportion to the passaging in cultures of CK cells the virus became more sensitive to heating at 50° for 30 minutes.

In the first 10-20 passages the virus formed large MC at 37°; by the 60th passage it had acquired the ability to form mc (with dimensions 1.5 mm) at 28°. Thus, the change in pathogenicity, ability to reproduce at high temperature, sensitivity to heating and dimensions of MC indicate further attenuation of the strain.

Testing of the virus in cattle showed that the method which we selected for attenuation of the virus in the organism of immune animals with subsequent passaging in a culture of CK cells at 24° made it possible to obtain a modified strain, which retained its immunogenic properties for cattle.

Markers used in the work for evaluating the degree of virulence of the strain make it possible to make preliminary conclusions about the degree of attenuation of the modified strain.

Conclusions

1. Hoof and mouth disease virus A_{22}, adapted over the course of 149 passages to the organism of the rabbit, as a result of further passaging in CK culture at 24 degrees lost the ability to propagate at an increased temperature and acquired the ability to propagate at low temperatures and to form small negative colonies, reduced the pathogenic properties for mice, rabbits, and guinea pigs.

2. The hoof and mouth disease virus strain obtained proved to be innocuous and immunogenic for cattle upon testing in preliminary experiments.

3. The methods used for evaluating the modified strain according to a series of markers proved to be suitable for determining the degree of attenuation of hoof and mouth disease virus A_{22}.

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13. ABSTRACT

Hoof and mouth disease virus A_{22}, adapted over the course of 149 passages to the organism of the rabbit, as a result of further passaging in CK culture at 24 degrees lost the ability to propagate at an increased temperature and acquired the ability to propagate at low temperatures and to form small negative colonies, reduced the pathogenic properties for mice, rabbits, and guinea pigs.

The hoof and mouth disease virus strain obtained proved to be innocuous and immunogenic for cattle upon testing in preliminary experiments.

The methods used for evaluating the modified strain according to a series of markers proved to be suitable for determining the degree of attenuation of hoof and mouth disease virus A_{22}.

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